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Prevalence of *Azospirillum* spp. in the rhizosphere of tropical plants

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Azospirillum cultures were isolated from the roots of a variety of tropical plants. Presumptive isolates from selective medium were identified by biochemical and serological methods. Forty-eight percent of the isolates were identified as *A. lipoferum* and the rest as *A. brasilense*, nir$^+$ or nir$^-$. All of the isolates tested exhibited at least one heat-labile antigen with one or more of the three whole-cell reference antisera. Isolates identified as *A. lipoferum* were very similar to the reference strain SP59, while those identified as *A. brasilense* showed a greater degree of antigenic and biochemical diversity. No cross-reaction was detected between the four reference antisera used and strains of *Azotobacter beijerinckia*, *A. chroococcum*, *A. vinelandii*, *Azomonas macrocytogenes*, and *A. agilis*.

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Des cultures d'*Azospirillum* ont été isolées des racines de diverses plantes tropicales. A partir du milieu sélectif, des isolats présomptifs ont été identifiés par des méthodes biochimiques et sérologiques. Quarante-huit pour cent des isolats ont été identifiés comme étant *A. lipoferum* et les autres comme étant *A. brasilense*, nir$^+$ ou nir$^-$. Tous les isolats que nous avons étudiés ont montré au moins un antigène thermolabile avec un ou plus des trois antisérums de référence de cellules entières. Les isolats identifiés comme étant *A. lipoferum* sont très similaires à la souche de référence SP59, tandis que les isolats qui ont été identifiés comme étant *A. brasilense* montrent un plus grand degré de diversité antigénique et biochimique. Aucune réaction croisée n'a été détectée entre les quatre antisérums de référence utilisés et les souches d'*Azotobacter beijerinckia*, *A. chroococcum*, *A. vinelandii*, *Azomonas macrocytogenes* et *A. agilis*.

[Traduit par le journal]

Nitrogen-fixing (diazotrophic) bacteria are widely distributed in nature. They have been reported to occur in a wide variety of habitats ranging from the polar regions to the tropics. Most notable among these are members of the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, and *Derxia* (for reviews see Day 1977; Neyra and Döbereiner 1977; van Berkum and Bohlool 1980). In particular, *Azospirillum* is the most common diazotroph associated with the roots of a variety of grasses (Day and Döbereiner 1976; Wong and Stenberg 1979; Starhorst and Strijdom 1978; Wong *et al.* 1980). Most investigators, however, consider this organism to be of potential significance only with tropical grasses (Day 1977; Neyra and Döbereiner 1977).

Biochemical and DNA homology study of this genus by Tarrand *et al.* (1978) resulted in the establishment of two species: *A. lipoferum* and *A. brasilense*. *Azospirillum brasilense* was further subdivided into two groups, nir$^+$ and nir$^-$, based on their ability to denitrify. The three groups can be differentiated by physiological criteria and DNA homology (Tarrand *et al.* 1978). Immunofluorescence (IF) and immunodiffusion (ID) studies have recently indicated antigenic differences as well (De-Polli *et al.* 1980; Schank *et al.* 1979). De-Polli *et al.* (1980) used immunofluorescence to categorize *Azospirillum* isolates on the basis of surface antigens and immunodiffusion to analyze the heat-labile and heat-stable diffusable components. At least four immunofluorescence serogroups were identified. One serogroup contained only members of *A. lipoferum*, while the other three fell within the *A. brasilense*, nir$^+$ and nir$^-$, subgroups. Immunodiffusion with whole-cell antisera resolved further differences among the isolates. However, there was close agreement between the two serogrouping methods. At least one heat-labile, gel-diffusable antigen was common to all of the 27 isolates of the three subgroups of *Azospirillum* tested (De-Polli *et al.* 1980).

In this study, we show that *Azospirillum* is present in the roots of tropical grasses (*Pennisetum clandestinum*, *P. setuceum*, *Paspalum urvillei*), trees (*Psidium Cattleianum* Sabine, *Pandanus tectorius*), pasture legumes (*Lotus Corniculatus* L.), herbaceous plants (*Hedychium coronarium*, *Cannabis sativa*, *Lantana camra* L.), ferns (*Athyrium* sp.), and club mosses (*Lycopodium cernum* L.), as well as in the rhizoids of a moss consortium. The *Azospirillum* isolates associated with these plants were characterized by the use of biochemical tests and by immunofluorescence and immunodiffusion.

Materials and methods

Azospirillum was isolated from surface-sterilized roots according to the methods of Döbereiner (1980). *Azospirillum* from moss were isolated by treating small pieces of the rhizoid in the same manner.

TABLE 1. Occurrence of *Azospirillum* in the roots of tropical plants

Scientific name	Common name	No. of isolates	Location on Island of:*
Psidium Cattleianum Sabine	Guava	1 (Gua 1)	Hawaii
Hedychium coronarium	Common gingerlily	1 (Ging 1)	Hawaii
Pandanus tectorius Sol.	Screwpine	5 (Pan 1–5)	Hawaii
Lotus Corniculatus L.	Birdsfoot trefoil	1 (Tre 1)	Hawaii
Pennisetum clandestinum	Kikuyugrass	1 (Kik 1)	Hawaii
Consortium from Class Musci	—	1 (Moss 1)	Hawaii
Cannabis sativa	Marijuana	3 (Can 1–3)	Oahu
Pennisetum setaceum	Fountaingrass	1 (FG 1)	Hawaii
Paspalum urvillei	Vaseygrass	1 (Pas 1)	Hawaii
Lantana camara L.	Lantana	1 (Lan 1)	Maui
Athyrium sp.	Ladyfern	1 (Ath 1)	Oahu
Lycopodium cernum L.	Nodding clubmoss	1 (Lyc 1)	Oahu

*The sites sampled are located on three of the six major islands that comprise the State of Hawaii, U.S.A.

The procedure used by Tarrand *et al.* (1978) was used for the biochemical tests. For immunofluorescence and immunodiffusion, the same antisera used by De-Polli *et al.* (1980) were applied. Gelatin – rhodamine isothiocyanate (Bohlool and Schmidt 1968) was used to suppress background fluorescence. Immunofluorescence and immunodiffusion procedures have been described elsewhere (De-Polli *et al.* 1980), except that cells for immunodiffusion were grown for 48 h in tryptone yeast broth (Hirsch *et al.* 1980).

Reference strains of *A. lipoferum* (Sp59) and *A. brasilense* nir$^+$ (SP7) and nir$^-$ (SP28 and SP107) were obtained from Dr. Johanna Döbereiner, Empresa Brasileira De Pesquisa Agropecuaria, Brazil. *Azotobacter* cultures of *A. beijerinckia* and *A. vinelandii* (two strains), and *Azomonas* cultures of *A. macrocytogenes* and *A. agilis* were obtained from Dr. E. L. Schmidt, University of Minnesota.

Results and discussion

Little is known about the geographical distribution of diazotrophic bacteria that associate with the roots of plants. A few, such as *Berjerinckia* and *A. paspalum*, are considered to have quite narrow ecological domains (Becking 1961; Döbereiner 1968; Neyra and Döbereiner 1977). In contrast, *Azospirillum* is considered to have a broader geographical and host-range distribution (Döbereiner *et al.* 1976; Okon *et al.* 1977; von Bülow and Döbereiner 1975). Nonetheless, *Azospirillum* has only received attention in its association with tropical grasses (Day 1977; Neyra and Döbereiner 1977).

In this study isolates of *Azospirillum* were found associated with the roots of a diverse group of tropical plants and the rhizoids of a moss consortium, from three of the major Pacific Islands that comprise the State of Hawaii, U.S.A. The host plant, location, and the number of isolates obtained from each host are given in Table 1.

The biochemical and morphological characteristics of the isolates and their reactions with fluorescent antibodies (FA) against reference strains of *Azospirillum* are presented in Table 2. All isolates could be grouped as *A. lipoferum*, *A. brasilense* (nir$^+$), or *A. brasilense* (nir$^-$) based on the criteria of Tarrand *et al.* (1978). All isolates, with the exception of two from *Pandanus* (Pan 3, Pan 5) and one from *Cannabis* (Can 2), fell within one of the four IF serogroups of De-Polli *et al.* (1980).

The isolates were further characterized by immunodiffusion on the basis of their gel-diffusible, whole-cell antigens. The isolates identified as *A. lipoferum* (Table 3) exhibited consistent and typical biochemical and serological (immunofluorescence and immunodiffu-

TABLE 2. Comparison of biochemical and immunological characteristics of *Azospirillum* isolates

Cultures	Species designation[a]	Immunofluorescence serogroup[b]
Reference strains		
SP 7	*A. brasilense* (nir$^+$)	SP 7
SP 28	*A. brasilense* (nir$^-$)	SP 28
SP 59	*A. lipoferum* (nir$^+$)	SP 59
SP 107	*A. brasilense* (nir$^-$)	SP 107
Isolates		
Gua 1	*A. lipoferum* (nir$^+$)	SP 59
Ging 1	*A. brasilense* (nir$^-$)	SP 28
Pan 1	*A. brasilense* (nir$^+$)	SP 107
Pan 2	*A. brasilense* (nir$^-$)	SP 7
Pan 3	*A. brasilense* (nir$^+$)	Negative
Pan 4	*A. brasilense* (nir$^+$)	SP 107
Pan 5	*A. brasilense* (nir$^+$)	Negative
Moss 1	*A. lipoferum* (nir$^-$)	SP 59
Tre 1	*A. lipoferum* (nir$^+$)	SP 59
Kik 1	*A. lipoferum* (nir$^+$)	SP 59
Can 1	*A. brasilense* (nir$^+$)	SP 107
Can 2	*A. brasilense* (nir$^+$)	Negative
Can 3	*A. lipoferum* (nir$^+$)	SP 59

[a]Based on criteria recommended by Tarrand *et al.* (1978).
[b]Based on the grouping of De-Polli *et al.* (1980).

TABLE 3. Immunodiffusion pattern of heat-labile and heat-stable (stomatic) soluble antigens of *Azospirillum* isolates

	Antisera[a]							
	Sp 7		Sp 28		Sp 59		Sp 107	
	whole	boiled	whole	boiled	whole	boiled	whole	boiled
A. lipoferum								
Sp 59	ab--	--	ab--	--	abcd	123	----	--
Moss 1	-b--	--	a---	--	ab--	---	----	--
Gua 1	----	--	----	--	abc-	---	----	--
Tre 1	a---	--	----	--	abc-	---	----	--
Kik 1	a---	--	a---	--	ab--	---	----	--
A. Brasilense (nir$^+$)								
Sp 7	abcd	12	a---	--	a-c-	---	----	--
Pan 1	abc-	1-	ab--	1-	----	---	----	--
Pan 3	abc-	1-	ab--	1-	----	---	a---	--
Pan 4	abc-	1-	ab--	-2	a---	---	a---	--
Pan 5	abc-	1-	ab-	-2	a---	---	a---	--
Can 1	abc-	1-	-b--	1-	-b--	---	a---	--
Can 2	abc-	1-	-b--	1-	-b--	---	a---	--
A. brasilense (nir$^-$)								
Sp 28	a---	--	abcd	12	a---	---	----	--
Sp 107	abc-	--	a---	--	ab--	---	ab--	1-
Ging 1	abc-	1-	ab--	1-	-b--	---	a---	--
Pan 2	abc-	1-	ab--	1-	----	---	----	--
Other diazotrophs[b]	----	--	----	--	----	---	----	--

[a]Antisera for Sp 7, Sp 28, and Sp 59 were prepared against unheated whole cells; antiserum for Sp 107 was prepared against boiled cells. The letters and the numbers refer to the precipitin bands produced with whole cells and heated cells, respectively, in the order of their position from the antibody well:

Antibody well | | | | Antigen well
a b c d
1 2 3

-, No band at that position.

[b]Other diazotrophs were *Azotobacter beijerinckia*, *A. chroococcum*, *A. vinelandii* (two strains), *Azomonas macrocytogenes*, and *A. agilis*.

sion) reactions. All of these strains (Gua 1, Moss 1, Tre 1, Kik 1, and Can 3) reacted strongly with *A. lipoferum* Sp 59 FA and formed two to three precipitin bands against Sp59 antiserum in immunodiffusion plates. None of them, however, were identical to the Sp59 strain by the pattern of their whole-cell or heat-stable gel-diffusible antigens.

Our data with the *A. brasilense* isolates revealed this group to be more diverse in their biochemical and serological characteristics. As shown in Table 3, several of these isolates reacted differently when compared by the two serological methods. The three isolates which did not react with any of the four fluorescent antibodies, Pan 3, Pan 5, and Can 2, all *A. brasilense* nir$^+$, had gel-diffusible antigens in common with both *A. brasilense* nir$^+$ (Sp7) and nir$^-$ (Sp 28). Other *A. brasilense* nir$^+$ isolates, Pan 1, Pan 4, and Can 1, fell within the Sp 7 group by immunodiffusion, while their cell-associated antigens were more closely related to the nir$^-$ Sp 107. Likewise, the *A. brasilense* nir$^-$ isolate Ging 1 reacted with the fluorescent antibody prepared against Sp 28, but its soluble antigens were more closely related to Sp 7, whereas isolate Pan 2, *A. brasilense* nir$^-$, had both surface and diffusible antigens more related to Sp7.

All of the isolates tested exhibited at least one heat-labile antigen with one or more of the three whole-cell reference antisera (Sp 7, Sp 28, and Sp 59). This common antigen could be demonstrated in all of the isolates, except in Gua 1 and Pan 2. Isolate Gua 1, identified as *A. lipoferum* (Sp 59), showed three heat-labile precipitin bands with the Sp 59 antiserum, but no bands with any of the other whole-cell antisera. Likewise, Pan 2, *A. brasilense* (nir$^-$), showed three bands and two bands with Sp 7 and Sp 28 antisera, respectively, but none with Sp 59. None of the four strains from the three different species of *Azotobacter* and the two species of *Azomonas* exhibited any detect-

able reaction against the three whole-cell (Sp 7, Sp 28, Sp 59) and the one somatic (Sp 107) antisera. This strengthens the suggestion implied from the results of De-Polli *et al.* (1980) that there may be a genus-specific antigen in *Azospirillum*.

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